

# Global Flexibility in a Sensory Receptor: A Site-Directed Cross-Linking Approach

JOSEPH J. FALKE AND DANIEL E. KOSHLAND, JR.

The aspartate receptor of *Escherichia coli* and *Salmonella typhimurium* is a cell surface sensory transducer that binds extracellular aspartate and sends a transmembrane signal to the inside of the bacterium. The flexibility and allostery of this receptor was examined by placing sulfhydryl groups as potential cross-linking sites at targeted locations in the protein. Seven different mutant receptors were constructed, each containing a single cysteine residue at a different position in the primary structure. Intramolecular disulfide bond formation within oligomers of these mutant receptors is shown to trap structural fluctuations and to detect ligand-induced changes in structure. The results indicate that the receptor oligomer has a flexible, dynamic structure which undergoes a global change upon aspartate binding.

TRANSMEMBRANE SIGNALING IS A WIDESPREAD PHENOMENON in biological systems. However, the mechanism by which external ligand binding generates an intracellular signal is poorly understood. A number of receptors have been proposed to contain only one or a few transmembrane peptide segments connecting the external and intracellular domains; such receptors include the insulin receptor (1, 2), the epidermal growth factor (EGF) receptor (3), the low-density lipoprotein (LDL) receptor (4), the nerve growth factor (NGF) receptor (5), and the aspartate and serine receptors of bacterial chemotaxis (6, 7). A wide range of models can be presented for the molecular mechanism of transmembrane signaling by these simple receptors. At one extreme, for example, a receptor could be a largely rigid structure in which ligand binding causes subtle shifts in the position of amino acid side chains, with only minor changes in the conformation of the polypeptide backbone. Alternatively, backbone flexibility may play an important role in transmembrane signaling, and in the limit of this case a receptor could be a highly dynamic structure that signals by a global, allosteric structural change involving large movements of its transmembrane segments and other polypeptide domains. We now report our results on the aspartate receptor of bacterial chemotaxis and their applicability to the study of protein flexibility and allostery.

A major difficulty in the analysis of transmembrane receptors is that physical studies of protein structure in the membrane are sometimes difficult to perform, while studies in solubilized systems raise questions as to whether detergent has altered the protein structure. Approaches that are equally applicable in membranes and

detergents are needed; the aspartate receptor of chemotaxis is particularly useful because it has been cloned (6), is easily altered by site-directed mutagenesis, and can be reintroduced into the living bacterium in order to test directly native structure and function. In addition, this receptor does not contain any cysteine residues; thus introduction of a single cysteine residue creates a new site at which the special chemistry of the sulfhydryl group can be utilized.

Construction and activity of cysteine-containing mutant receptors. We used site-directed mutagenesis to construct seven different mutant receptors, each containing a single cysteine residue at a different designated position within the molecule. The sites were chosen to maximize the probability of yielding sulfhydryl groups on the surface of active receptors. Therefore, the cysteines were substituted near charged residues, at positions that are unconserved in the five known bacterial chemoreceptor sequences (6-9). Four cysteine residues were placed near the ends of the two known transmembrane segments (6, 10). Two were placed in the external domain where aspartate binding is known to occur, and one in the carboxyl-terminal domain where methylation and intracellular signaling occur (6, 8, 11, 12). The positions of cysteine substitution are summarized in Fig. 1A.

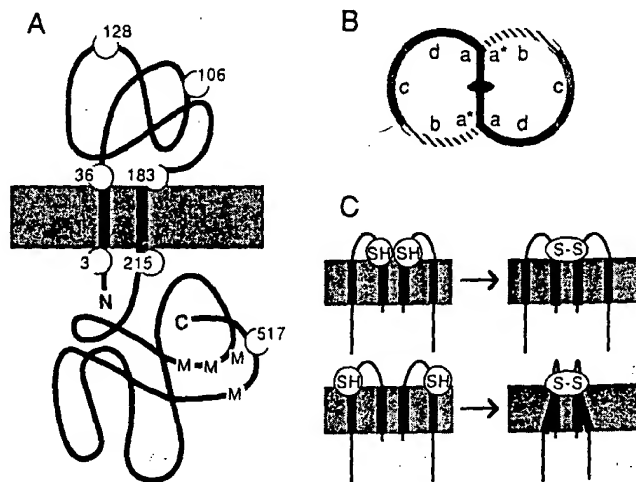
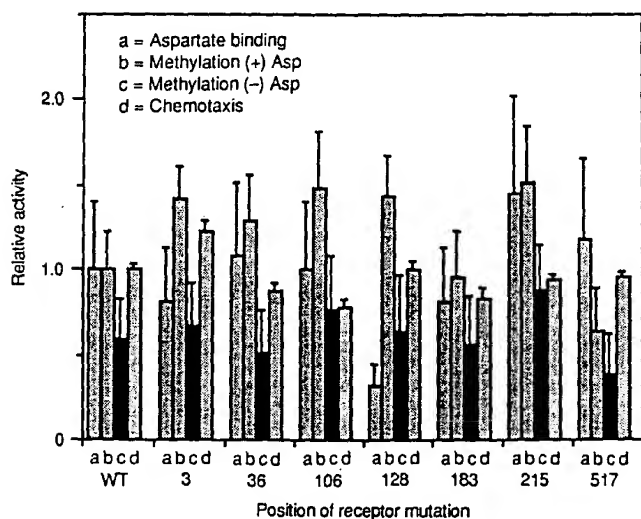


Fig. 1. Model for aspartate receptor structure. (A) The proposed arrangement of the aspartate receptor in the membrane showing the intracellular amino terminus, two membrane-spanning segments, the intracellular methylation sites (M), and the numbered positions of cysteine substitution (circles). (B) A hypothetical oligomeric structure, in which identical monomers are arranged around a  $C_2$  axis (center), with four regions on each monomer indicated (a to d). Contact between monomers is at region a, a\*. (C) Disulfide formation within a dimer of identical monomers, in one case involving minimal distortion and in a second case involving substantial distortion.

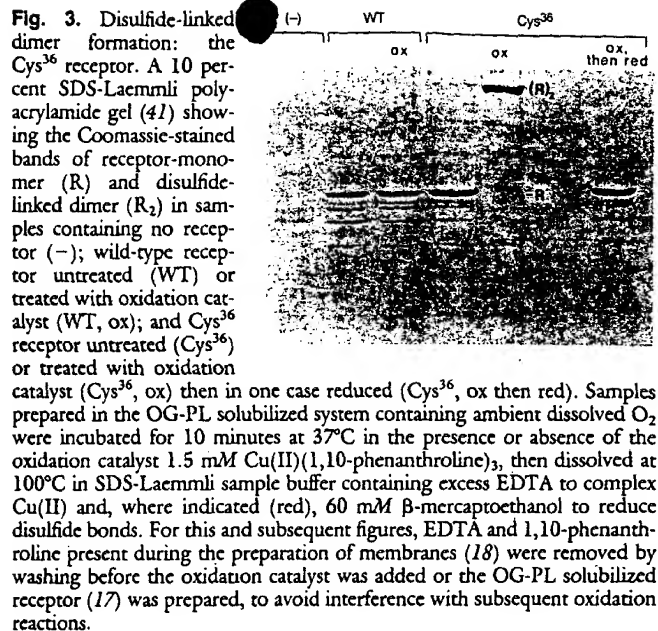
The authors are in the Department of Biochemistry, University of California Berkeley, Berkeley, CA 94720.

Mutagenesis was performed in an M-13 vector according to the oligonucleotide-directed method as modified by Kunkel (13). Successful substitution of a cysteine codon at the desired location was confirmed by dideoxy sequencing (14). The wild-type or mutant gene, together with the natural promoter, was then cloned into the pEMBL 18 plasmid (15), which was used to transform the gene into *Escherichia coli* (16). The activity of each mutant receptor was tested by analysis of its aspartate binding, regulation, and signal transduction characteristics. Each of the membrane-bound mutant receptors exhibits an aspartate binding constant similar to that of the wild-type receptor (Fig. 2). When the mutant receptors are solubilized, partially purified, and assayed in an OG-PL solubilized system described previously [0.39 percent (w/v) octylglucoside, 0.10 percent *E. coli* lipid, and 19 percent glycerol (17)], their regulatory glutamate residues are methylated by the methyltransferase enzyme at rates essentially identical to that of the wild-type receptor in the same system. Moreover, each of the mutant receptors exhibits an aspartate-induced increase in methylation rate which is essentially identical to that of the wild-type receptor (Fig. 2). Finally, when the mutant genes are transformed into an *E. coli* strain that lacks the aspartate receptor, each of the mutant receptors restores the chemotactic swarming of the transformed bacteria to a rate that is within a factor of 1.5 that observed for the wild-type receptor (Fig. 2). It follows that each of the mutant receptors containing substituted cysteine residues retains a fully intact, functional structure.

**Disulfide cross-linking.** The seven different cysteine-containing receptors have been used to subject the receptor structure to a sensitive test for flexibility. The receptor is an oligomer in octylglucoside (18–20), and disulfide bond formation between cysteines on different monomers has proved useful in the detection of structural fluctuations since such fluctuations are required to bring distant



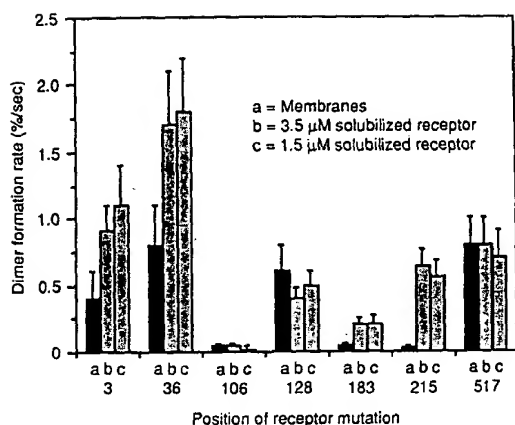
**Fig. 2.** Comparison of the activities of wild-type and mutant receptors. The pEMBL 18 plasmid containing the wild-type or mutant *Salmonella typhimurium* aspartate receptor gene was transformed into *E. coli* RP3808 (16), a strain lacking the receptor as well as the methyltransferase and esterase, for studies (a to c) and subsequent figures; and into *E. coli* RP 4372 (16), a strain lacking the receptor, for study (d). Measured as previously described (17, 33, 40) were (a) the aspartate binding constant of receptor in isolated membranes ( $K_B \pm SD = (8 \pm 3) \times 10^5 M^{-1}$  for wild-type receptor); (b) and (c) the rate of transferase-catalyzed methylation of regulatory glutamate residues on receptor in the OG-PL solubilized system ( $[^3H]$ methyl addition rate =  $0.11 \pm 0.03$  Me/(receptor  $\cdot$  min) for wild-type receptor + 1 mM aspartate); and (d) the chemotactic swarm rate of transformed bacteria on tryptone soft agar (swarm rate =  $1.27 \pm 0.04$  mm/hour for RP4372 transformed with wild-type receptor). Activities in each case are plotted relative to the indicated wild-type receptor activity. Error bars are  $\pm 1$  standard deviation for  $n \geq 3$ , simultaneous experiments.



cysteines into contact (see below). Disulfide formation was observed (Fig. 3) when the receptor was oxidized by ambient oxygen at 22°C with Cu(II)(1,10-phenanthroline)<sub>3</sub> as a catalyst (21). To stop the reaction at specific times, we denatured the oligomer in the presence of EDTA [to chelate Cu(II)] and *N*-ethylmaleimide (to block free sulfhydryl groups); the products were then analyzed by SDS-polyacrylamide gel electrophoresis (PAGE). A typical result is shown in Fig. 3 for the Cys<sup>36</sup> receptor, which upon oxidation migrates quantitatively as a disulfide-cross-linked dimer on SDS-PAGE. When oxidation is followed by reduction with  $\beta$ -mercaptoethanol, the Cys<sup>36</sup> receptor migrates quantitatively as a monomer, demonstrating that the dimer linkage is in fact a simple disulfide bond. Densitometric analysis of the monomer and dimer bands yields the fraction of receptor in the disulfide-linked dimeric state as a function of reaction time. The initial rates of disulfide-linked dimer formation for each of the seven cysteine-containing receptors in both the membrane and solubilized states are summarized in Fig. 4. In every case a measurable rate is observed within the range 0.05 to 1.7 percent disulfide-linked dimer formed per second.

In order to interpret disulfide reaction rates in terms of receptor flexibility, it is important to ascertain whether the observed disulfide reactions occur intramolecularly within the receptor oligomer, or intermolecularly through collisions between oligomers. To resolve these possibilities, several types of disulfide reactions have been carried out. The products of these reactions can be complex, and the following nomenclature is used to distinguish the products. The position of a cysteine is indicated by a number, and cysteines on different peptide chains are distinguished by a prime. Thus *n-m* represents a disulfide bond between cysteines at positions *n* and *m* on the same receptor monomer, while *n-m'* represents a disulfide bond between cysteines *n* and *m* on different monomers. For example, 3-215' represents a disulfide bond between Cys<sup>3</sup> on one monomer and Cys<sup>215</sup> on a second monomer. Since each aspartate receptor monomer is composed of a single polypeptide chain, *n-m'* yields a disulfide-linked dimer on denaturing gels. Three types of reactions have proved useful in the resolution of intra- and intermolecular disulfide bond formation.

1) If disulfide bond formation occurs within the oligomer, then the size of the oligomer should be unaffected by the reaction. If it occurs by collision between oligomers, the products should have a larger molecular size. When the size of the detergent-solubilized

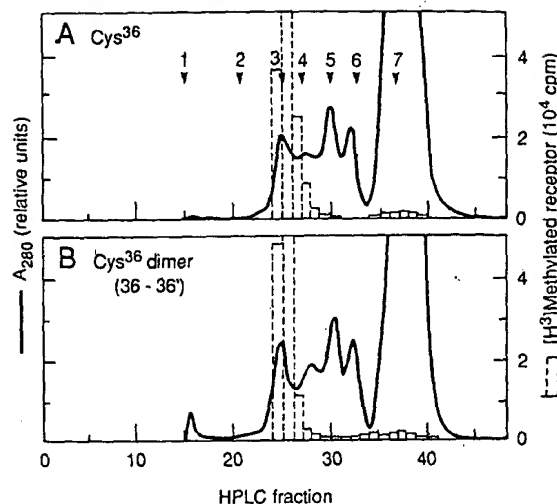


**Fig. 4.** Initial disulfide-linked dimer formation rates of the cysteine receptors. The indicated receptors were prepared in both membrane and OG-PL solubilized systems, and disulfide formation was initiated by addition of oxidation catalyst at 22°C (Fig. 3). At specific times, samples were removed, and the reaction was quenched by addition of double-strength Laemmli gel sample buffer containing 4 percent SDS to denature the receptor oligomer, 5 mM EDTA to chelate Cu(II), and 10 mM *N*-ethylmaleimide to block free sulfhydryl groups; the reaction was then heated immediately to 100°C for 3 minutes. Subsequently the extent of disulfide-linked dimer formation was determined by SDS-polyacrylamide gel electrophoresis, including densitometric analysis of the receptor monomer and dimer bands [extent dimer formation =  $R_2/(R + R_2)$ ; see Fig. 3]. Initial rates were determined from the slope of the best-fit straight line to the early time points in each reaction. Error bars are +1 standard deviation for  $n \geq 3$  simultaneous experiments.

Cys<sup>36</sup> oligomer is examined by high-performance liquid chromatography (HPLC) gel filtration, an apparent molecular size of 230 kD is obtained both before and after quantitative formation of the 36–36' disulfide bond (Fig. 5), indicating that disulfide formation has no effect on the oligomeric state.

2) For disulfide formation within the oligomer, the reaction rate should be independent of receptor concentration, while for disulfide formation between oligomers, the rate should be sensitive to concentration. When mutant receptors are diluted by orders of magnitude by solubilization from the membrane, or when they are subsequently diluted in the solubilized system, their *n*-*n'* disulfide formation rates are not decreased (Fig. 4). Thus rate-limiting collisions between oligomers are not involved in disulfide bond formation.

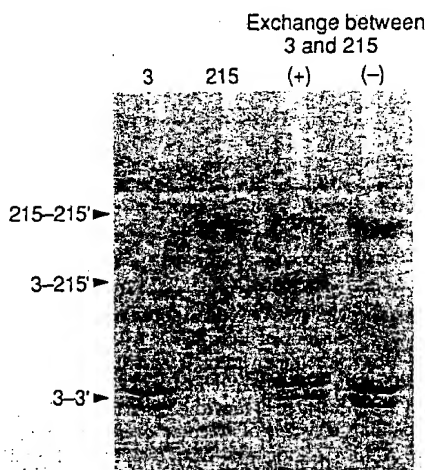
3) In order to test directly for inter-oligomer disulfide formation, we made use of the observation that different disulfide-linked dimers migrate differently on SDS-polyacrylamide gels (Fig. 6). This property can be utilized to test whether a disulfide bond is formed between two monomers within the same oligomer (intramolecular), or between a monomer in one oligomer and a monomer in a second oligomer (intermolecular) [protocol of Milligan and Koshland (22)]. If the disulfide cross-linking occurs only intramolecularly and there is no exchange of subunits between oligomers, then a mixture of Cys<sup>3</sup> oligomers and Cys<sup>215</sup> oligomers will produce only 3–3' or 215–215' disulfide-linked dimers. If, however, collisional intermolecular cross-linking occurs, 3–215' disulfide-linked dimers as well as 3–3' and 215–215' disulfide-linked dimers should be found. As can be seen in Fig. 6, no 3–215' disulfide-linked dimers are observed when Cys<sup>3</sup> oligomers are mixed with Cys<sup>215</sup> oligomers and disulfide formation is initiated. One might argue that there is an inherent kinetic barrier to 3–215' bond formation, due to insufficient reactivity or proximity. To examine this possibility, we incubated the homo-oligomers under conditions that promote exchange of monomers between oligomers until hetero-oligomers are formed, containing both Cys<sup>3</sup> and Cys<sup>215</sup> monomers within the same oligomer. When these hetero-oligomers are then subjected to



**Fig. 5.** Effect of disulfide-linked dimer formation on oligomer size. HPLC gel filtration profiles of solubilized Cys<sup>36</sup> receptor oligomers methylated with [<sup>3</sup>H]methyl groups as previously described (11). Methylated receptor oligomers were solubilized from membranes with 1 percent  $\beta$ -octylglucoside in the (A) absence or (B) presence of oxidation catalyst at 0°C (Fig. 3). After the extraction, 2.5 mM *N*-ethylmaleimide; 5 mM EDTA, and 2.5 mM PMSF (phenylmethylsulfonyl fluoride) were added to each sample, and samples were analyzed by gel filtration in 100 mM NaH<sub>2</sub>PO<sub>4</sub>, 1 percent nononyl-*N*-methylglucamide, pH 7. The (A) reduced and (B) disulfide-linked receptors in the radioactive fractions were verified by SDS-polyacrylamide gel electrophoresis. The molecular size standards are (2) 669 kD; (5) 43 kD; (6) 17 kD; and (7) 1.3 kD. The apparent molecular size are (1) void volume (receptor incubated with antibody to receptor migrates here); (3) 230 kD, receptor oligomer; (4) 93 kD; trypsin-treated receptor oligomer (12).

oxidation, 3–215' disulfide-linked dimers are observed (Fig. 6). Similar experiments with other pairs of mutants [Cys<sup>36</sup> with Cys<sup>183</sup> (22), and Cys<sup>106</sup> with Cys<sup>183</sup>] have given similar results, indicating that the intramolecular nature of disulfide formation is not limited to a particular pair.

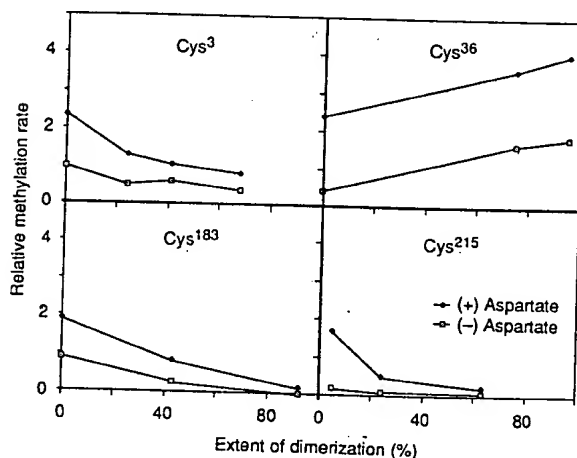
These three independent approaches indicate that each of the observed disulfide reactions occurs exclusively by an intramolecular reaction, that is, exclusively by a reaction cross-linking two different monomers within the same oligomer.



**Fig. 6.** Disulfide-linked heterodimer formation. SDS-polyacrylamide gels of disulfide-linked dimers produced by reaction of the following. Cys<sup>3</sup> receptor only (first column); Cys<sup>215</sup> receptor only (second column); Cys<sup>3</sup> and Cys<sup>215</sup> receptors mixed under exchange conditions (third column); and Cys<sup>3</sup> and Cys<sup>215</sup> receptors mixed under non-exchange conditions (fourth column). Samples containing 1 percent  $\beta$ -octylglucoside and the indicated receptor oligomers were incubated at 37°C for 5 minutes. Subsequently the receptors were diluted tenfold into the OG-PL solubilized system containing 1 mM aspartate, which stops exchange (22), and, in one case, the homo-oligomers were then mixed (fourth column). Disulfide-linked formation was catalyzed at 37°C for 10 minutes; the reaction was quenched and the products were analyzed (Fig. 4). Bands appear as doublets due to minor proteolysis during the incubations.

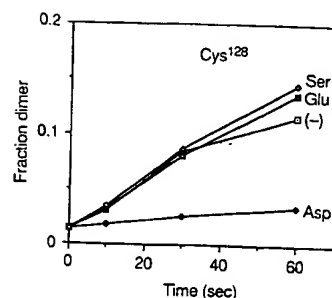
**Disulfide cross-linking as a probe for flexibility.** The nature of the flexibility required for the formation of intra-oligomer disulfide bonds is illustrated in Fig. 1B. The HPLC gel filtration data (Fig. 5) indicates that the oligomer has an apparent molecular size of 230 kD; a similar result was obtained earlier (18). Since the known molecular size of the monomer is 59 kD (6), and since monomers can be quantitatively converted to disulfide-linked dimers within the oligomer (Figs. 3 to 6), it follows that the receptor is a dimer or a tetramer. The simplest case of a dimeric structure requires the presence of a twofold rotation axis at the center of the average structure, normal to the membrane. In such a case, a cysteine will most often be distant from a second cysteine at the same position in a different monomer, relative to the 2 Å disulfide bond length. Examples are cysteines in regions b, c, and d of Fig. 1B. For a cysteine in any of these regions, considerable fluctuation of the receptor structure away from its average state is required to bring the cysteine close to the corresponding cysteine on the neighboring monomer. Smaller fluctuations are required for a cysteine in the contact region a,a\* of Fig. 1B, since positions that lie near the central axis are close to the corresponding positions on other monomers. However, even for a cysteine in region a,a\* some degree of structural fluctuation will generally be needed for a-a\* disulfide bond formation.

The rates of intra-oligomer disulfide formation vary widely between the different cysteine mutants. The fastest rate is obtained for 36-36' formation, which exhibits an initial rate of 1.7 percent dimer formed per second in the solubilized oligomer, while the slowest case, 106-106' formation, has an initial rate of  $\leq 0.05$  percent dimer formed per second (Fig. 4). The divergent rates are consistent with the idea that disulfide formation is sensitive to factors that differ for the different cysteine mutants, including the proximity of reactive cysteines in the average structure, the frequen-



**Fig. 7.** Effect of disulfide-linked dimer formation on receptor methylation activity. Solubilized receptor was oxidatively dimerized as indicated, and the rate of methylation of the regulatory glutamate residues of the receptor by methyltransferase was determined in the OG-PL solubilized system (17) in the absence or presence of 1 mM L-aspartate. The rate of methylation is calculated relative to that of wild-type receptor in the same system, lacking aspartate. Varying extents of disulfide dimerization were obtained by exposure to different oxidation conditions (0 to 1.5 mM catalyst, 0 to 37°C, 15 to 30 minutes) and subsequent addition of reducing agent to quench oxidation (0 to 25 mM dithioerythritol, 22° to 37°C, 15 to 30 minutes). The final extent of the disulfide-linked dimer formation was determined as in Fig. 4 except that all electrophoresis samples contained 25 mM NaAsO<sub>2</sub>, which tightly complexes dithioerythritol but not monothiolols (42) to prevent further reduction. Control experiments indicated that under these conditions NaAsO<sub>2</sub> itself has no effect on disulfide formation, and that the different oxidation and reduction conditions used have no effect on the methylation rate of the wild-type receptor. Points are averages of two independent experiments with differences less than 11 percent.

**Fig. 8.** Effect of aspartate on disulfide-linked dimer formation in the solubilized Cys<sup>128</sup> receptor. The time course of disulfide-linked dimer formation was measured in the OG-PL solubilized system (Fig. 4), in the absence of any added amino acid (-), or in the presence of 1 mM L-aspartate, L-glutamate, or L-serine. Shown is a time course from a single experiment; the analogous experiment in the membrane system yielded the same result.

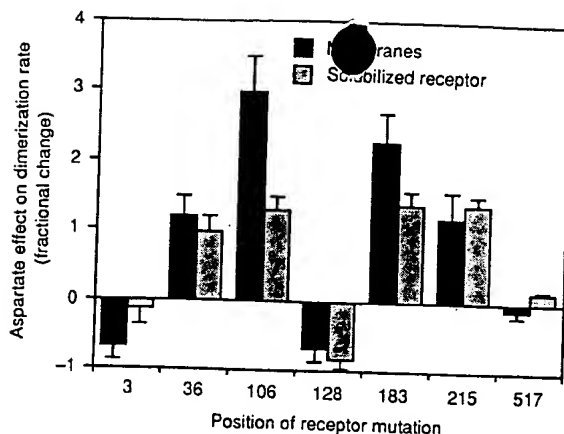


cy of structural fluctuations that bring distant cysteines into contact, and the inherent reactivity of the sulfhydryl group in a given environment. The increase in n-n' disulfide formation rates on solubilization for the membrane-bordering Cys<sup>3</sup>, Cys<sup>36</sup>, Cys<sup>183</sup>, and Cys<sup>215</sup> residues indicates that the proximity, mobility, or reactivity of these cysteines increases when the steric constraints of the bilayer are removed. In addition, the detection of disulfide formation for cysteine positions distributed throughout the primary structure indicates that substantial, widespread flexibility is present within the receptor oligomer (Figs. 1 and 4). Limited flexibility in the structure of folded proteins has been previously indicated by NMR, hydrogen-exchange, fluorescence, and x-ray diffraction measurements (23-29), and by theoretical calculations (30, 31). The disulfide bond formation technique can be used to trap covalently a wide variety of structural fluctuations, including new types that are otherwise undetectable because of low occupancy, such as short-lived fluctuations involving large amplitude deviations from an average structure.

Fluctuations trapped by disulfide bond formation can be identified by their effect on receptor activity. If the trapped structure represents a significant deviation from the average structure, then the receptor will be locked in a conformed, inactive state. The effect of disulfide formation on the activity of the solubilized receptor was tested by measuring receptor methylation in the absence and presence of aspartate. In previous studies this has been a critical test for the native structure of the receptor (17). We now find that methylation of the Cys<sup>36</sup> receptor is slightly activated by 36-36' disulfide formation and retains an aspartate effect, while other mutant receptors are inactivated by n-n' disulfide formation (Fig. 7). This result suggests that the Cys<sup>36</sup> residue lies near the central axis, where it rapidly participates in disulfide formation without significantly disrupting the active structure (Fig. 1C, upper). In contrast, the Cys<sup>3</sup>, Cys<sup>183</sup>, and Cys<sup>215</sup> residues are distant from the central axis, and in each case n-n' disulfide formation traps an inactive fluctuation significantly different from the active structure (Fig. 1C, lower).

**Disulfide cross-linking as a probe for allostery.** Disulfide formation can also be used to probe the effects of ligand on receptor structure. Aspartate is known to bind to the outside of the receptor and to transmit a signal to the intracellular compartment (32, 33). Such a signal must involve some change in the structure of the receptor, but the extent of this change is unknown. The seven cysteine-containing mutant receptors can be used to ascertain whether ligand binding induces a localized or rather a global change in the receptor conformation. When the control ligands glutamate and serine, which bind weakly or not at all to the aspartate receptor, respectively (33), are added prior to initiation of the disulfide reaction, there is little or no effect on the rate of n-n' disulfide formation (Fig. 8). However, when aspartate is added, a large change occurs in the rate of n-n' disulfide formation for many of the cysteine positions (Figs. 8 and 9). Some rates increase, some rates decrease, and others remain relatively unchanged. This result is





**Fig. 9.** Effect of aspartate on initial disulfide-linked dimer formation rates. Rates of disulfide-linked dimer formation were measured in the membrane and OG-PL solubilized systems (Fig. 4) in the absence (v) or presence (v') of 1 mM L-aspartate. The fractional change in dimerization rate is defined as  $(v' - v)/v$ . In some cases (Cys<sup>3</sup>, Cys<sup>128</sup>, and Cys<sup>516</sup> receptors) the initial disulfide-linked dimer formation rates were varied by slowing the reaction through addition of 0 to 5 mM EDTA, without significant alteration of the relative change due to aspartate. Error bars are  $\pm 1$  standard deviation for  $n \geq 3$  independent measurements.

direct evidence for a global conformational change affecting a large portion of the structure of a receptor during transmembrane signaling. The large magnitude of many of the observed changes reemphasizes the sensitivity of disulfide formation rates to structural and environmental changes. Different regions of the molecule behave differently. Many simple explanations, such as a monotonic global increase or decrease in flexibility, or a rigid oligomer which signals by subtle shifts in amino acid side chains, are excluded by this evidence. Furthermore, the effect of aspartate on disulfide formation rates is similar in the solubilized and membranes systems (Fig. 9), supporting the conclusion that the solubilized receptor in the mixed micelle system has an essentially native structure (17).

**Conformational flexibility and allostery.** The evidence obtained by site-directed cross-linking indicates that (i) the aspartate receptor oligomer exhibits flexibility of substantial range and amplitude both in the absence and presence of aspartate and (ii) transmembrane signaling by this receptor involves a global change in the structure of the oligomer. This is consistent with the biological function of the receptor since external binding of aspartate triggers changes affecting both the internal signaling site and the internal adaptation site of the protein (20). In view of the similarity in structure of the aspartate receptor to other receptors such as the insulin, EGF, LDL, and NGF receptors it seems plausible that these sensory transducers may generally have flexible structures that undergo global conformational changes during transmembrane signaling. An important question for further study is whether unusual flexibility is a universal feature of receptors and sensory transducers, and whether they undergo global conformational changes involving key or all transmembrane segments. One of the useful features of site-directed cross-linking is that it provides a generalizable tool for analyzing and comparing the flexibility and conformational changes of these and other proteins in vivo, in membranes, and in solubilized systems.

The use of cysteine as a probe in protein systems has many potential applications. Because of the ease of localized mutagenesis, a cysteine residue can now be placed in essentially any selected region of a cloned protein. Our approach involving substitution of cysteine for unconserved residues will be useful for many studies; in other studies cysteines can be substituted for semi- or strictly conserved residues. Similarly, our use of cysteine mutagenesis and interpeptide disulfide formation to study flexibility and conforma-

tional changes in oligomeric proteins is only one possible application. Cysteine mutagenesis and intra-peptide disulfide formation have also been used to study pathways of protein folding (34), the thermal stability and proteolytic resistance of proteins (35-37), and the geometry and energetics of disulfide bonds (36, 38). Such intra-peptide disulfide bonds can further be used to "freeze" a chosen region in the activated or inactivated regulatory state. Both inter- and intra-peptide disulfide reactions can trap structural fluctuations in folded proteins that comprise too small a fraction of the total population of conformations to be observed by spectroscopic and diffraction techniques. In addition, because cysteines can be placed by mutagenesis at any two points within a protein, disulfide formation can be used to develop quantitative maps of structural fluctuations.

Other important applications of cysteine mutagenesis include the introduction of sulfhydryl groups into proteins at key locations as chemical and spectroscopic labeling sites. The rate of reaction of sulfhydryl groups with chemical modification agents, and the spectroscopic properties of modified sulfhydryls, are expected to be sensitive indicators of sulfhydryl reactivity, proximity, and conformational dynamics; these independent methods will be useful in dissecting the separate roles of reactivity, proximity, and dynamics during disulfide formation. In short, site-directed cysteine mutagenesis can be used to investigate flexibility, induced conformational changes, and other static and dynamic features of protein structure.

#### REFERENCES AND NOTES

1. A. Ullrich *et al.*, *Nature (London)* 313, 756 (1985).
2. Y. Ebina *et al.*, *Cell* 40, 747 (1985).
3. A. Ullrich *et al.*, *Nature (London)* 309, 418 (1984).
4. T. Yamamoto *et al.*, *Cell* 39, 27 (1984).
5. M. J. Radeke *et al.*, *Nature (London)* 325, 593 (1987).
6. A. F. Russo and D. E. Koshland, Jr., *Science* 220, 1016 (1983).
7. A. Boyd, K. Kendall, M. I. Simon, *Nature (London)* 301, 623 (1983).
8. A. Krikos, N. Mutoh, A. Boyd, M. I. Simon, *Cell* 33, 615 (1983).
9. J. Bollinger *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 81, 3287 (1984).
10. C. Manoil and J. Beckwith, *Science* 233, 1403 (1986).
11. T. C. Terwilliger and D. E. Koshland, Jr., *J. Biol. Chem.* 259, 7719 (1984).
12. S. L. Mowbray, D. L. Foster, D. E. Koshland, Jr., *ibid.* 260, 11711 (1985).
13. T. A. Kunkel, *Proc. Natl. Acad. Sci. U.S.A.* 82, 488 (1985).
14. F. Sanger, S. Nicklen, A. R. Coulson, *ibid.* 74, 5463 (1977).
15. L. Dente, G. Cesarine, R. Cortese, *Nucleic Acids Res.* 11, 1645 (1983).
16. RP4372 is a gift from J. S. Parkinson, University of Utah, Salt Lake City.
17. E. Bogonez and D. E. Koshland, Jr., *Proc. Natl. Acad. Sci. U.S.A.* 82, 4891 (1985).
18. D. L. Foster *et al.*, *J. Biol. Chem.* 260, 11706 (1985).
19. D. Chelsky and F. W. Dahlquist, *Biochemistry* 19, 4633 (1980).
20. R. M. Macnab, in *E. coli and S. typhimurium*, F. C. Neidhardt, Ed. (American Society for Microbiology, Washington, DC, 1987), vol. 1, p. 732.
21. K. Kobashi, *Biochim. Biophys. Acta* 158, 239 (1968).
22. D. M. Milligan and D. E. Koshland, Jr., in preparation.
23. J. Ygeurabide, H. F. Epstein, L. Stryer, *J. Mol. Biol.* 51, 573 (1970).
24. S. W. Englander *et al.*, *Annu. Rev. Biochem.* 41, 903 (1972).
25. J. R. Lakowicz and G. Weber, *Biochemistry* 12, 4171 (1973).
26. J. C. Norvell, A. C. Nunes, B. P. Schoenborn, *Science* 190, 568 (1975).
27. G. Wagner, H. Tschesche, K. Wütrich, *Eur. J. Biochem.* 95, 239 (1979).
28. H. Frauenfelder *et al.*, *Nature (London)* 280, 558 (1979).
29. T. A. Steitz *et al.*, *Phil. Trans. R. Soc. B323*, 43 (1981).
30. M. Karplus and J. A. McCammon, *Annu. Rev. Biochem.* 53, 263 (1983).
31. R. Elber and M. Karplus, *Science* 235, 318 (1987).
32. M. L. Toes, M. F. Goy, M. S. Springer, J. Adler, *Proc. Natl. Acad. Sci. U.S.A.* 76, 5544 (1979).
33. S. Clarke and D. E. Koshland, Jr., *J. Biol. Chem.* 254, 9695 (1979).
34. T. E. Creighton, *Methods Enzymol.* 131, 83 (1986).
35. C. B. Marks, H. Naderi, P. A. Kosen, I. D. Kuntz, S. Anderson, *Science* 235, 1370 (1987).
36. L. J. Perry and R. Wetzel, *ibid.* 226, 555 (1984).
37. J. A. Wells and D. B. Powers, *J. Biol. Chem.* 261, 6564 (1986).
38. L. J. Perry and R. Wetzel, *Biochemistry* 25, 733 (1986).
39. B. A. Katz and A. Kossiakoff, *J. Biol. Chem.* 261, 15480 (1986).
40. J. Adler, *Science* 153, 708 (1966).
41. U. K. Laemmli, *Nature (London)* 227, 680 (1970).
42. W. L. Zahler and W. W. Cleland, *J. Biol. Chem.* 243, 716 (1968).
43. We thank A. F. Demburg, D. W. Sternberg, and N. Zalkin for technical assistance, and D. M. Milligan for helpful discussions. Supported by grants from the National Institutes of Health and the National Science Foundation (D.E.K.), and by a National Institutes of Health postdoctoral fellowship (J.J.F.).

14 May 1987; accepted 27 August 1987.